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Pentamidine-Induced Long QT Syndrome and Block of HERG Trafficking

[1]Yuri A Kuryshev, [1]Eckhard Ficker, [1]Lu Wang, [2]Peter Hawryluk, [1]Adrienne T Dennis, [1,2]Barbara A Wible, [1,2]Arthur M Brown, [3]Jiesheng Kang, [3]Xiao-Liang Chen, [3]Kaoru Sawamura, [3]William Reynolds and [3]David Rampe

[1]RammelkampCenter for Education and Research, MetroHealth Campus, Case Western Reserve University, Cleveland Ohio (YAK, EF, LW, ATD, BAW, AMB), [2]ChanTest Inc, Cleveland, Ohio (PH, BAW, AMB) and [3]Drug Safety Evaluation, Aventis Pharmaceuticals Inc, Bridgewater, NJ (JK, X-LC, KS, WR, DR)

Running title: Pentamidine-induced block of hERG trafficking

Corresponding authors:

Eckhard Ficker, Rammelkamp Center, MetroHealth Medical Center, 2500 MetroHealth Dr., Cleveland, OH 44109. Tel.: 216-778-8977; Fax: 216-778-8282

Email: eficker@metrohealth.org

Arthur M Brown, ChanTest Inc., 14656 Neo Parkway, Cleveland, OH 44128. Tel.: 216-332-1665; Fax: 216-332-1706

Email: abrown@chantest.com

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Abbreviations: APD, action potential duration, hERG, human ether a-go-go-related gene, I_{Kr}, rapidly activating delayed rectifier K current, pentamidine, 1,5-Bis(p-amidinophenoxy)pentane

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Abstract

The diamidine pentamidine is used to treat leishmaniasis, trypanosomiasis and *Pneumocystis carinii* pneumonia. Treatment may be accompanied by prolongation of the QT interval of the electrocardiogram and torsades de pointes tachycardias. Up to now it has been thought that therapeutic compounds causing QT prolongation are associated with direct block of the cardiac potassium channel hERG, which encodes the α-subunit of cardiac I_{Kr} currents.

We show that pentamidine has no acute effects on currents produced by hERG, KvLQT1/mink, Kv4.3 or *SCNA5*. Cardiac calcium currents and the guinea pig cardiac action potential were also not affected. After overnight exposure, however, pentamidine reduced hERG currents and inhibited trafficking and maturation of hERG with IC_{50}s of 5-8μM similar to therapeutic concentrations. Surface expression determined in a chemiluminesence assay was reduced on exposure to 10, 30 and 100μM pentamidine by about 30, 40 and 70%, respectively. These effects were specific for hERG since expression of hKv1.5, KvLQT1/minK and Kv4.3 was not altered. In isolated guinea pig ventricular myocytes, 10 μM pentamidine prolonged action potential duration APD_{90} from 374.3 ± 57.1 to 893.9 ± 86.2ms on overnight incubation. I_{Kr} tail current density was reduced from 0.61 ± 0.09 to 0.39 ± 0.04pA/pF. We conclude that pentamidine prolongs the cardiac action potential by block of hERG trafficking and reduction of the number of functional hERG channels at the cell surface. We propose that pentamidine like arsenic trioxide produces QT prolongation and torsades de pointes in patients by inhibition of hERG trafficking.

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Introduction

The antiprotozoal agent pentamidine isethionate is used in developing countries for the treatment of parasitic diseases such as trypanosomiasis and antimony-resistant visceral leishmaniasis (Burchmore et al., 2002; Nacher et al., 2001). In the US it is used in the treatment of *Pneumocystis carinii* pneumonia, a common opportunistic infection in patients who have contracted the human immunodeficiency virus or in patients immunosuppressed during chemotherapy (Sands, et al., 1985; Goa and Campoli-Richards, 1987). Therapy with pentamidine is often accompanied by prolongation of the QT interval on the electrocardiogram (ECG) and, in some instances, by torsades de pointes tachycardias (TdP) that can degenerate into ventricular fibrillation and cause sudden cardiac death (Wharton et al., 1987; Bibler et al., 1988; Girgis et al., 1997).

Prolongation of the QT interval and torsades de pointes are usually seen in patients with inherited (congenital) long QT syndrome (Keating and Sanguinetti, 2001) or in association with a wide variety of structurally diverse medications including antiarrhythmic, antihistamine, antibiotic and psychotropic compounds (Fermini and Fossa, 2003). Most drugs known to cause acquired long QT syndrome do so by direct blockade of the cardiac potassium channel hERG (Redfern et al., 2003, Pearlstein et al., 2003), which underlies the rapid component of the delayed rectifier potassium current I_{Kr} in human heart (Sanguinetti et al., 1995; Keating and Sanguinetti, 2001). While some of these drugs such as class III antiarrhythmics were designed to prolong cardiac repolarization with hERG as the intended target (Vaughn Williams et al., 1982), for the vast majority, block of hERG constitutes an unwanted adverse side effect. To determine drug-related cardiac toxicity HERG block is usually detected directly by patch clamp electrophysiology on the cloned hERG channel. Although hERG/I_{Kr} is most extensively studied,

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other cardiac potassium currents e.g. the slow component of the delayed rectifier current I_{Ks} (encoded by KvLQT1/minK), the ultra-rapidly activating delayed rectifier current I_{Kur} (encoded by Kv1.5) or the transient outward current I_{to} (encoded by Kv4.3) may provide additional plausible substrates for acquired long QT syndrome.

Recently, we have reported a completely different mechanism associated with acquired long QT syndrome and TdP. We have shown that arsenic trioxide (As_2O_3) used in the treatment of acute promyelocytic leukemia reduced hERG/I_{Kr} currents not by direct block, but by inhibiting the processing of hERG protein in the endoplasmic reticulum (ER) thereby decreasing surface expression of hERG (Ficker et al., 2004). Another compound that reduces hERG currents by trafficking inhibition is geldanamycin, a benzoquinoid antibiotic that specifically inhibits function of the cytosolic chaperone Hsp90 (Ficker et al., 2003). Derivatives of geldanamycin are in clinical trials for the treatment of various forms of cancer with no reports of adverse cardiac events currently available. In the present report, we examine a novel compound class represented by the antiprotozoal agent pentamidine which has been associated clinically with QT prolongation and TdP and show that the aromatic diamidine pentamidine acts via inhibition of hERG channel trafficking.

Methods

Cellular electrophysiology-HEK293, L-cells or Chinese hamster ovary (CHO) cells stably expressing hERG, hKv1.5, KvLQT1/minK, or Kv4.3 potassium channels were studied using the whole cell configuration of the patch clamp technique. Patch pipettes were filled with (in mM): K-aspartate, 100, KCl, 20, $MgCl_2$, 2, $CaCl_2$, 1, EGTA, 10, HEPES 10 (pH7.2). The extracellular solution was (in mM): NaCl, 140, KCl, 5, $MgCl_2$, 1, $CaCl_2$, 1.8, HEPES 10,

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glucose, 10 (pH 7.4). Stably transfected cells lines used in this study were regularly checked for responses to positive control compounds: cisapride was used for hERG; 4-aminopyridine (4-AP) for hKv1.5; chromanol 293B for KvLQT1/mink; flecainide for Kv4.3 and lidocaine for *SCNA5* expressing cells. In guinea pig ventricular myocytes nisoldipine was used to validate L-type calcium currents. To study delayed effects of pentamidine on heterologously expressed hERG, hKv1.5, KvLQT1/minK and Kv4.3 potassium currents, drug was added to stable cell lines for 16-20 hrs (overnight) prior to recording. Sodium currents were recorded in HEK293 cells stably expressing the human cardiac Na^+ channel gene *SCN5A* using the following extracellular solution (in mM): NaCl, 40, NMDG, 55, CsCl, 20, KCl, 5.4, $MgCl_2$, 2, $CaCl_2$, 0.02, TEA Cl, 30, 4-AP, 5, HEPES, 10 (pH 7.4). The intracellular solution was (in mM): CsCl, 120, $MgCl_2$, 2, $CaCl_2$, 1, EGTA 11, HEPES 10, glucose 10, Mg-ATP, 1 (pH 7.2). Cardiac L-type calcium currents were recorded in freshly isolated guinea pig ventricular myocytes using the following extracellular solution (in mM): NaCl, 137, CsCl, 5.4, $MgCl_2$, 1.8, $CaCl_2$, 1.8, glucose, 10, HEPES, 10 (pH 7.4). The intracellular solution was (in mM): Cs $MeSO_4$, 130, TEA Cl, 20, $MgCl_2$, 1, EGTA 10, HEPES 10, Mg-ATP, 4, Tris-phosphocreatine, 14, Tris-GTP, 0.3, creatine phosphokinase 50U/ml (pH 7.2). Action potentials and cardiac I_{Kr}/hERG currents were recorded in ventricular guinea pig myocytes either freshly isolated or cultured overnight in M199 medium using the following intracellular solution (in mM): K-gluconate, 119, KCl, 15, $MgCl_2$, 3.75, EGTA, 5, HEPES, 5, K-ATP, 4, phosphocreatine, 14, Tris-GTP, 0.3 and 50U/ml creatine phosphokinase (pH 7.2). The extracellular solution was (in mM): NaCl, 132, KCl, 4, $MgCl_2$, 1.2, $CaCl_2$, 1.8, HEPES 5 (pH 7.4). Measurements of cardiac I_{Kr} currents were performed in the presence of 1μM nisoldipine to block L-type Ca^{2+} currents. The specific blocker E4031 was used to pharmacologically isolate I_{Kr}. To analyze current densities, membrane capacitances were

measured using the analogue compensation circuit of an Axon 200B patch clamp amplifier (Axon Instruments, Foster City, CA). pCLAMP software (Axon Instruments) was used to generate voltage-clamp protocols and for data acquisition. All recordings were performed at room temperature (20-22^{o}C). Data are expressed as means ± SEM.

Western blot analysis-The HEK/hERG cell line, the L/hKv1.5 cell line and antibodies used in the present study have been described previously (Ficker et al., 2003)). Briefly, stably transfected cells expressing either hERG or hKv1.5 were solubilized for 1h at 4^{o}C in a lysis buffer containing 1% Triton X-100 and protease inhibitors (Complete, Roche Biochemicals, Indianapolis, IN). Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Proteins were separated on SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes and developed using appropriate antibodies and ECL Plus (Amersham Biosciences, Piscataway, NJ). For quantitative analysis, chemiluminescence signals were captured directly on a Storm Phosphoimager (Amersham Biosciences). Normalized image densities are expressed as means ± SEM.

Chemiluminescence detection of cell surface hERG protein-A hemagglutinin (HA) tag was inserted into the extracellular loop of hERG between transmembrane domains S1 and S2 (Ficker et al., 2003). Stably transfected HEK/hERG WT HA_{ex} cells were plated at 40,000 cells/well in a 96-well plate. After overnight incubation with pentamidine, cells were fixed with ice-cold 4% paraformaldehyde, blocked by incubation with 1% goat serum and incubated for one hour with rat anti-HA antibody (Roche Diagnostics). After washing, HRP-conjugated goat anti-rat IgG (Jackson Labs, West Grove, PA) and the dsDNA stain SYBR Green (Molecular Probes, Eugene, OR) were added for one hour (Myers, 1998; Margeta-Mitrovic et al., 2000). SYBR Green fluorescence was measured to determine cell numbers. Chemiluminescent signals were

developed using SuperSignal (Pierce) and captured in a luminometer. If necessary luminescence signals in pentamidine treated wells were corrected for cell loss as measured by SYBR Green fluorescence with the data presented as normalized surface expression relative to control (means ± SEM). For correction of cell loss a standard curve of SYBR Green fluorescence was generated using four different amounts of cells per well (10, 20, 30 or $40*10^3$, n=3).

Results

Pentamidine does not directly block hERG currents. Direct block of the cardiac potassium channel hERG is the most common mechanism underlying acquired long QT syndrome. Therefore we first studied the effects of acute application of extracellular pentamidine on hERG currents activated with depolarizing pulses to +20mV from a holding potential of –80mV. Pentamidine block was evaluated by analyzing peak tail current amplitudes on return to –40mV (Fig. 1A). Pentamidine demonstrated no significant inhibition of hERG currents with ascending concentrations ranging from 0.3 to 10 μM (Fig 1B). With 10 μM pentamidine in the extracellular perfusate we observed a maximum inhibition of 13 ± 3%, which was similar to current run-down observed over the same time period (12 ± 5%, n=6). To exclude the possibility that access of the dicationic pentamidine to an intracellular blocking site was hindered, we performed experiments with 10μM pentamindine added to the intracellular pipette solution. With intracellular application of pentamidine, hERG currents showed a small time-dependent run-down averaging 14 ± 5% when measured 10min after initiation of whole cell recordings (n=4). This value was similar to that observed for vehicle-treated (0.1% DMSO) control cells (15 ± 5% reduction after 10min, n=3).

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Since block of other repolarizing cardiac potassium currents such as Kv4.3 or KvLQT1/minK may also prolong the cardiac action potential (Nerbonne, 2000), we tested whether those currents were affected by pentamidine. Both KvLQT1/minK and Kv4.3 channels were activated by depolarizing step pulses to +20mV from a holding potential of –80mV. Currents were continuously recorded while 10μM pentamidine was applied for 10min with the extracellular bath solution. Neither KvLQT1/minK nor Kv4.3 currents were blocked at this concentration ($p>0.05$, paired t-test; n=5-6; Fig 2A and B). In addition, we studied a possible contribution of cardiac inward currents to the proarrhythmic effects exerted by pentamidine. We elicited sodium currents in HEK293 cells stably expressing the cardiac sodium channel gene *SCN5A* using depolarizing pulses to –20mV from a holding potential of –110mV while perfusing 10μM pentamidine for 10min. At this concentration neither peak currents nor current kinetics were affected ($p>0.05$, paired t-test, n=6, Fig 2C). Since cardiac calcium currents are composed of multiple subunits and are not easily reconstituted in heterologous expression systems, we used freshly isolated guinea pig ventricular myocytes to evaluate pentamidine effects. Cardiac calcium currents were activated from a holding potential of –40mV with depolarizing step pulses to 0mV. In these experiments we detected a small reduction in peak current amplitudes of 4.4 ± 3.7% (n=3) while steady state currents remained stable in the presence of 10μM pentamidine (Fig 2D).

Prolonged exposure to pentamidine inhibits maturation of hERG. Since pentamidine did not directly block cardiac membrane currents, we explored whether prolonged exposure to pentamidine might interfere with hERG processing as reported for the anti-neoplastic drugs geldanamycin (Ficker et al., 2003) and As_2O_3 (Ficker et al., 2004). To this end, we exposed stably transfected HEK/hERG cells overnight (16-20hrs) to increasing concentrations of

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pentamidine. We found that hERG currents were reduced in a dose-dependent manner with an IC_{50} of 5.1 μM as determined by analyzing changes in tail current amplitudes (n=8-10, Fig 3A and B). To validate our electrophysiological analysis we performed Western blots of hERG protein isolated under control conditions and after overnight exposure to increasing concentrations of pentamidine. HERG channels are synthesized as a core-glycosylated, immature ER form of about 135kDa and as a mature, fully-glycosylated cell surface form of about 160kDa. Incubation with pentamidine produced a dose-dependent decrease in the amount of mature fully-glycosylated hERG protein (Fig 4A). Expression of the mature, cell surface form of hERG was suppressed with an IC_{50} of 7.8μM (n=3; Fig 4B), which is similar to the IC_{50} of 5.1μM determined in electrophysiological experiments on chronic exposure to pentamidine. To quantify pentamidine-induced changes in surface expression of hERG protein more directly, we used a chemiluminescence assay. This assay was performed with HEK293 cells stably expressing a modified hERG protein with an extracellular hemagglutinin (HA) epitope tag inserted in the extracellular S1-S2 linker (Ficker et al., 2003). HEK/hERG-HA_{ex} cells were treated overnight with 10, 30μM or 100μM pentamidine and surface expression of hERG-HA_{ex} was reduced by 30, 40 and 70%, respectively (Fig 4C).

Specificity of pentamidine effects on hERG trafficking. Since processing of hERG may be handled by proteins shared between different ion channels, the question arises whether the observed pentamidine effect is specific for hERG or whether other cardiac potassium channel are affected in a similar manner. To test for specificity, we exposed L-cells stably expressing the ultrarapid delayed rectifier hKv1.5 overnight (16-20hrs) to increasing concentrations of pentamidine and performed Western blots. For all concentrations tested we detected hKv1.5 protein as a core-glycosylated, immature ER form of about 68kDa and as a mature, fully-

glycosylated cell surface form of about 75kDa (Fig 5A). Incubation with pentamidine did not alter the expression pattern of hKv1.5 (Fig 5B). In line with our biochemical data, we found that hKv1.5 current densities were not significantly altered upon overnight incubation with 10μM pentamidine (Fig 4C). We measured 636.6 ± 60pA/pF under control conditions and 625.6 ± 135.5pA/pF after overnight exposure to pentamidine ($p>0.05$, Student's t-test, n=6). We also recorded currents from HEK 293 cells stably expressing KvLQT1/minK and Kv4.3 channels under control conditions and after overnight exposure to pentamidine (Fig 6Aa, 6Ba) and found for both channels that current densities were not altered ($p>0.05$, Student's t-test). For KvLQT1/minK we measured current densities of 40.5 ± 8.7pA/pF (n=6) under control conditions and of 48.1 ± 13.2pA/pF (n=8) after overnight exposure to 10 μM pentamidine (Fig 6Ab). In Kv4.3 expressing cells we measured 240 ± 38.5pA/pF (n=16) under control conditions and 195.7 ± 88.3pA/pF (n=10) in the presence of 10 μM pentamidine (Fig 6Bb).

Prolonged exposure to pentamidine prolongs the cardiac action potential and reduces I_{Kr} in guinea pig ventricular myocytes. To test whether our results obtained in heterologous expression systems can also be applied to cardiomyocytes, we studied the effects of pentamidine on the cardiac action potential. Acute application of 10 μM pentamidine failed to alter action potentials measured in freshly isolated guinea pig ventricular myocytes (Fig 7). In these experiments APD_{90} was 535 ± 32ms under control conditions (0min) and 522 ± 31ms after extracellular perfusion of 10 μM pentamidine. Action potentials were further studied by culturing myocytes overnight in the absence or presence of 10 μM pentamidine. We found that 10 μM pentamidine prolonged APD_{90} significantly from 374.3 ± 57.1 to 893.9 ± 86.2ms ($p<0.05$; Student's t-test, n=10-11, Fig 8A and B). This indicated that chronic drug exposure

induces changes compatible with clinically observed QT prolongation and TdP. Since our experiments in heterologous expression systems pointed towards a reduction of the native I_{Kr}/hERG current as a possible cause for the observed action potential prolongation, we determined I_{Kr} current densities in voltage clamp experiments performed in guinea pig ventricular myocytes. I_{Kr} currents were elicited in myocytes cultured overnight using a ramp protocol and isolated as E4031 sensitive tail current component upon return to -40mV (Fig 8C). In these experiments chronic exposure to 10 μM pentamidine significantly reduced I_{Kr} tail current density by about 35% from 0.61 ± 0.09 to 0.39 ± 0.04pA/pF ($p<0.05$, Student's t-test, n=6-7, Fig 8D).

Discussion

This report is the first to describe the effects of the antiprotozoal drug pentamidine on cardiac ion channels. Pentamidine is known to produce QT prolongation and TdP in clinical use. Most drugs that produce adverse cardiac events do so via a direct block of the cardiac potassium channel hERG that is easily detected with patch clamp techniques (Redfern et al., 2003, Pearlstein et al., 2003). We were, therefore, surprised to find that acute administration of pentamidine had no immediate effect on hERG currents. Similarly, acute administration of pentamidine failed to affect four different, major cardiac ion channels including KvLQT1/minK, Kv4.3, *SCN5A* Na^+ channels and L-type calcium channels. By contrast, prolonged treatment of hERG-expressing cells resulted in a dose-dependent reduction of hERG currents with an IC_{50} of about 5 μM. The effect was specific since current densities of heterologously expressed hKv1.5, KvLQT1/minK and Kv4.3 channels were not altered. Western blots revealed that the reduction in hERG current density was associated with a decrease in the fully glycosylated cell surface

form of the hERG protein. Accordingly, I_{Kr} density was reduced and the cardiac action potential was prolonged in cardiomyocytes. Based on these data, we propose that QT prolongation and ventricular tachycardias observed in patients treated with pentamidine are not caused by direct block of hERG or other cardiac ion channels, but rather are the result of a reduction in I_{Kr} current density due to an acquired trafficking block of hERG/I_{Kr} channels.

Pentamidine has been widely used in the treatment of *Pneumocystis carinii* pneumonia in patients infected with human immunodeficiency virus (Goa and Campoli-Richards, 1987). The drug is also used in developing countries to treat a variety of parasitic diseases including trypanosomiasis and leishmaniasis (Burchmore et al., 2002; Nacher et al., 2001). Typically the drug is administered via daily intramuscular injection or slow intravenous infusion at a dose of 4 mg/kg of body weight. These doses result in peak serum levels that range from about 1-5 μM (Sands et al., 1985, Conte et al., 1986; Lidman et al., 1994), similar to the concentrations required for inhibition of hERG trafficking *in vitro*. Prolongation of the QT interval on the electrocardiogram and the development of TdP tachycardias are well-documented adverse events associated with pentamidine treatment (Wharton et al., 1987; Bibler et al., 1988; Girgis et al., 1997; Kroll and Gettes, 2002). However, QT prolongation is not immediately evident in these patients and generally takes several days to develop (Stein et al., 1991; Eisenhauer et al., 1994; Otsuka et al., 1997). We believe that this slow time course is consistent with a pentamidine-induced decrease in the expression of functional hERG channels in the heart, rather than a direct blocking effect of the drug, since QT prolongation by direct hERG channel blockers such as dofetilide are evident immediately upon administration (Lande et al., 1998).

At present it is not clear how pentamidine interferes with hERG processing and maturation. In microbial cells pentamidine has been reported to inhibit topoisomerase activity

and decrease intracellular ATP content while in mammalian cells it has been shown to inhibit several tyrosine phosphatases (Reddy et al., 1999; Pathak et al., 2002). Other antimicrobial agents such as quinolone antibiotics, which also function as topoisomerase inhibitors in microbial systems, have been shown to directly block hERG currents (Cirioni et al., 1997; Larsen et al., 2003; Anderson et al., 2001). Similarly, the antineoplastic topoisomerase inhibitor amsacrine, which has been associated with adverse cardiac events, reduces hERG currents by direct block (Thomas et al., 2004). Both quinolone antibiotics (sparfloxacin, ofloxacin and ciprafloxacin) and amsacrine do not affect hERG trafficking (unpublished data). Given these observations we speculate that the pentamidine-induced trafficking block of hERG is not due to inhibition of topoisomerases but rather to inhibition of a protein or proteins required for successful maturation of hERG potassium channels. Further studies will be necessary to elucidate the biochemical mechanism(s) responsible for pentamindine's effects on hERG protein trafficking.

In summary, the present study demonstrates that the proarrhythmic effects of pentamidine are not due to a direct blockade of hERG currents, but are consistent with inhibition of hERG trafficking and a reduction in the number of functional hERG channels in the heart. Thus, pentamidine joins arsenic trioxide, another non anti-arrhythmic compound that is proarrhythmic as a result of an acquired hERG trafficking defect. Geldanamycin, an anti-neoplastic Hsp90 inhibitor currently in clinical trials, may be proarrhythmic for similar reasons (Ficker et al., 2004; Ficker et al., 2003). In this regard chemotherapeutic agents that disrupt protein synthesis as their intended mechanism of action may be of special concern. Likewise, drugs that produce QT prolongation in the clinic only after prolonged treatment may suggest similar effects on the processing of ion channel proteins. At present, the detection of cardiac risk as a result of

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acquired trafficking defects has not been considered and the hERG assay presently recommended by the International Conference of Harmonization[1] will fail to identify trafficking inhibitors.

References

Anderson ME, Mazur A, Yang T and Roden DM (2001) Potassium current anatagonist properties and proarrhythmic consequences of quinolone antibiotics. J Pharmacol Exp Ther **296**: 806-810.

Bibler MR, Chou TC, Toltzis RJ and Wade PA (1988) Recurrent ventricular tachycardia due to pentamidine-induced cardiotoxicity. *Chest* **94**: 1303-1306.

Burchmore RJS, Ogbunude POJ, Enanga B and Barrett MP (2002) Chemotherapy of human African trypanosomiasis. *Curr Pharm Des* **8**: 256-267.

Cirioni O, Giacometti A, Quarta M and Scalise G (1997) In vitro activity of topoismerase inhibitors against *Pneumocystis carinii*. *J Antimicrob Chemother* **40**, 583-586.

Conte JE Jr, Upton RA, Phelps RT, Wolfsy CB, Zurlinden E and Lin ET (1986) Use of a specific and sensitive assay to determine pentamidine pharmacokinetics in patients with AIDS. *J Infect Dis* **154**: 923-929.

Eisenhauer MD, Eliasson AH, Taylor AJ Coyne PE and Wortham DC (1994) Incidence of cardiac arrhythmias during intravenous pentamidine therapy in HIV-infected patients. *Chest* **105**: 389-395.

Fermini B and Fossa AA (2003) The impact of drug-induced QT interval prolongation on drug discovery and development. *Nat Rev Drug Discov* **2**: 439-447.

Ficker E, Dennis AT, Wang L, and Brown AM (2003) Role of the cytosolic chaperones Hsp70 and Hsp90 in maturation of the cardiac potassium channel HERG. *Circ Res* **92**: e87-100.

Ficker E, Kuryshev YA, Dennis AT, Obejero-Paz C, Wang L, Hawryluk P, Wible BA and Brown AM (2004) Mechanisms of arsenic-induced prolongation of cardiac repolarization. *Mol Pharmacol*, **66**: 33-44.

Girgis I, Gualberti J, Langan L, Malek S, Mustaciuolo V, Costantino T and McGinn TG (1997) A prospective study of the effect of IV pentamidine therapy on ventricular arrhythmias and QTc prolongation in HIV-infected patients. *Chest* **112**: 646-653.

Goa KL and Campoli-Richards DM (1987) Pentamidine isethionate. A review of its antiprotozoal activity, pharmacokinetic properties and therapeutic use in *Pneumocystis carinii* pneumonia. *Drugs* **33**: 242-258.

Keating MT and Sanguinetti MC (2001) Molecular and cellular mechanisms of cardiac arrhythmias. *Cell* **104**: 569-580.

Kroll CR and Gettes LS (2002) T wave alternans and Torsades de Pointes after the use of intravenous pentamidine. *J Cardiovasc Electrophysiol* **13**: 936-938.

Lande G, Maison-Blanche P, Fayn J Ghadanfar M, Coumel P and Funck-Brentano C (1998) Dynamic analysis of dofetilide-induced changes in ventricular repolarization. *Clin Pharmacol Ther* **64**: 312-321.

Larsen AK, Escargueil AE, Skladanowski A (2003) Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther* **99**: 167-181.

Lidman C, Bronner U, Gustafsson LL and Rombo L (1994) Plasma pentamidine concentrations vary between individuals with Pneumocystis carinii pneumonia and the drug is actively secreted by the kidney. *J Antimicrob Chemother* **33**: 803-810.

Margeta-Mitrovic M, Jan YN, and Jan LY (2000) A trafficking checkpoint controls $GABA_B$ receptor heterodimerization. *Neuron* **27**: 97-106.

Myers MA (1998) Direct measurement of cell numbers in microtitre plate cultures using the fluorescent dye SYBR green I. *J Immunol Methods* **212**: 99-103.

Nacher M, Carme B, Sainte-Marie D, Couppie P, Clyti E, Guibert P and Pradinaud R (2001) Influence of clinical presentation on the efficacy of a short course of pentamidine in the treatment of cutaneous leishmaniasis in French Guiana. *Ann Trop Med Parasitol* **95**: 331-336.

Nerbonne JM (2000) Molecular basis of functional voltage-gated K^+ channel diversity in the mammalian myocardium. *J Physiol* **525**: 285-298.

Otsuka M, Kanamori H, Sasaki S, taguchi J, Harano H, Ogawa K, Matsuzaki M, Mohri H, Okubo T, Sumita S and Ochiai H (1997) Torsades de Pointes complicating pentamidine therapy of *Pneumocystis Carinii* pneumonia in acute myelogenous leukemia. *Intern Med* **36**: 705-708.

Pathak MK, Dhawan D, Lindner DJ, Borden EC, Farver C and Yi T (2002) Pentamidine is an Inhibitor of PRL phosphatases with anticancer activity. *Mol Cancer Ther* **1**: 1255-1264.

Pearlstein R, Vaz R and Rampe D (2003) Understanding the structure-activity relationship of the human ether-a-go-go-related gene cardiac K+ channel. A model for bad behavior. *J Med Chem* **46**: 2017-2022.

Reddy BS, Sondhi SM and Lown JW (1999) Synthetic DNA minor groove-binding drugs. *Pharmacol Ther* **84**: 1-111.

Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, Siegl PKS, Strang I, Sullivan AT, Wallis R, Camm AJ, and Hammond TG (2003) Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. *Cardiovasc Res* **58**: 32-45.

Sands M, Kron MA and Brown RB (1985) Pentamidine: a review. *Rev Infect Dis* **7**: 625-634.

Sanguinetti MC, Jiang C, Curran ME, and Keating MT (1995) A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{Kr} potassium channel. *Cell* **81**: 299-307.

Stein KM, Fenton C, Lehany AM, Okin PM and Kligfield P (1991) Incidence of QT interval prolongation during pentamidine therapy of *Pneumocystis Carinii* pneumonia. *Am J Cardiol* **68**: 1091-1094.

Thomas D, Hammerling BC, Wu k, Wimmer A-B, Ficker EK, Kirsch GE, Kochan MC, Wible BA, Scholz EP, Zitron E, Kathofer S, Kreye VAW, Katus HA, Schoels W, Karle CA and Kiehn J (2004) Inhibition of cardiac HERG currents by the DNA topoisomerase II inhibitor amsacrine: mode of action. *Br J Pharmacol*, **142**: 485-94.

Vaughn Williams, EM, Millar, JS and Campbell T J (1982) Electrophysiological effects of labetolol on rabbit atrial, ventricular and Purkinje cells, in normoxia and hypoxia. *Cardiovasc. Res.* **16**, 233-239.

Wharton JM, Demopulos PA and Goldschlager N (1987) Torsade de Pointes during administration of pentamidine isethionate. *Am J Med* **83**: 571-576.

Footnotes

[1] ICH S7B-The nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. European Medicines Agency EMEA. CHMP/ICH/423/02 (June 2004).

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Legends for figures

Figure 1. Effects of pentamindine on hERG currents. ***A,*** whole-cell hERG currents were elicited by 2-s depolarizing pulses to +20 mV from a holding potential of –80 mV at 40-s intervals. The membrane potential was then returned to –40 mV to generate large outward tail currents. The effects of 3 and 10 μM pentamindine are shown. ***B,*** dose-response relationship of pentamidine block. Pentamidine produced 13 ± 3% inhibition of hERG currents at the highest concentration tested (10 μM, n=6), a value similar to the current run-down observed over the same time period (approximately 10 minutes).

Figure 2. Pentamidine has no effect on other major cardiac membrane currents. ***A***, Heterologously expressed KvLQT1/minK currents recorded under control conditions and after extracellular application of 10 μM pentamidine. Pentamidine had no effect on KvLQT1/minK (n=5). ***B***, Effects of pentamidine on heterologously expressed Kv4.3 currents and ***C,*** cardiac Na^+ currents (expressed from *SCN5A* gene) recorded under control conditions and in the presence of 10 μM pentamidine. As in the case of KvLQT1/minK, 10 μM pentamidine has no effect on either Kv4.3 or Na^+ currents (n=6). ***D***, effect of 10 μM pentamidine on native L-type calcium currents recorded in guinea pig ventricular myocytes. Exposure to 10 μM pentamidine failed to significantly reduce cardiac Ca^{2+} currents (n=3). Voltage protocols for each experiment are shown above current traces.

Figure 3. Prolonged exposure to pentamidine suppresses hERG currents heterologously expressed in HEK293 cells. ***A***, representative hERG current recordings obtained under control

conditions (*left panel*) and from an HEK/hERG cell incubated overnight with 10 μM pentamidine (*right panel*). Currents were elicited from a holding potential of -80 mV with pulses from -60 to +60 mV in 20 mV increments. Tail currents were recorded on return to -50 mV. ***B***, concentration-dependence of pentamidine effect (24 h treatment). IC_{50} is 5.1 μM (n=8-10).

Figure 4. Pentamidine inhibits maturation of hERG. ***A***, Western blot showing effects of overnight treatment with pentamidine on hERG channel protein stably expressed in HEK293 cells. On Western blots pentamidine reduces fully glycosylated, mature 160 kDa hERG in a concentration-dependent manner. Treatment with 10 μM of As_2O_3 was used as positive control. ***B***, concentration-dependent reduction of fully glycosylated 160kDa hERG after overnight exposure to pentamidine. Image densities on Western blots were quantified using Storm PhosphoImager and normalized to control. IC_{50} is 7.8 μM (n=3). ***C***, overnight treatment with pentamidine reduces in a concentration-dependent manner surface expression of HA_{ex}-tagged hERG protein stably expressed in HEK293 cells as determined by chemiluminesence measurements. Surface expression levels were normalized relative to control.

Figure 5. Pentamidine does not alter the expression pattern of hKv1.5. ***A***, Western blot showing steady-state levels of hKv1.5 protein stably expressed in L-cells after prolonged exposure to increasing concentrations of pentamidine. HKv1.5 is expressed as a fully-glycosylated protein of 75 kDa and as a core-glycosylated protein of 68 kDa. ***B***, image densities of fully glycosylated and core-glycosylated hKv1.5 protein were quantified as a function of pentamidine concentrations using a PhosphorImager. All image densities were normalized to the

fully-glycosylated 75 kDa protein form measured under control conditions (n=3). ***C***, hKv1.5 current densities measured after overnight culture under control conditions and in the presence of 10μM pentamidine (n=6). Current densities are presented in statistical box charts and were not significantly different (Student's T test). In box charts asterisks represent outliers, whiskers determine the 5^{th} and 95^{th} percentiles, boxes determine the 25^{th} and 75^{th} percentiles and means are represented by square symbols.

Figure 6. KvLQT1/minK and Kv4.3 channels are not sensitive to overnight treatment with 10 μM pentamidine. ***A,*** KvLQT1/minK channels stably expressed in HEK293 cells. ***Aa***, representative current recordings obtained from control (left) and pentamidine treated (right) cell. Currents were elicited from a holding potential of -80 mV with 2 s pulses from -60 to +20 mV in 20 mV increments. Tail currents were recorded on return to -50 mV. ***Ab***, current density measured in cells cultured overnight under control conditions and in the presence of 10 μM pentamidine. Current densities were determined from current amplitudes at +20 mV and normalized to cell capacitance. Data are presented in statistical box charts as described in the legend to Figure 5. ***B***, Kv4.3 channels stably expressed in HEK293 cells. ***Ba***, representative current recordings obtained from control (left) and pentamidine treated (right) cells. Currents were elicited from a holding potential of -80 mV with depolarizing 400ms voltage steps from -60 to +20 mV in increments of 20 mV. ***Bb***, Current densities were measured in the absence and presence of 10μM pentamidine as peak currents at 0 mV and normalized to cell capacitance. Data are presented in statistical box charts.

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Figure 7. Acute application of pentamidine does not prolong cardiac action potentials. Cardiac action potentials elicited in a freshly isolated ventricular myocyte immediately after establishing whole-cell configuration (0min) and 5min after start of extracellular perfusion with 10μM pentamidine. Membrane potential was –80mV.

Figure 8. Prolonged exposure to pentamidine prolongs cardiac action potentials and reduces I_{Kr} in cultured guinea pig ventricular myocytes. ***A***, representative cardiac action potentials recorded from ventricular myocytes cultured overnight (24hr) under control conditions (left panel) and after incubation with 10μM pentamidine (right panel). ***B***, Action potential duration (APD_{90}) measured in control and pentamidine treated (10 μM; 24 hr) cardiomyocytes. ***C***, isolation of E4031-sensitive I_{Kr} current in a ventricular myocyte cultured overnight. Current traces were elicited with a 1sec voltage ramp from –80 to +80mV. Holding potential was –80mV. I_{Kr} currents were isolated as E4031-sensitive tail current component on return to –40mV. E4031-sensitive subtraction currents are shown in right part of panel for a control myocyte and for a myocyte cultured overnight in the presence of 10μM pentamidine. Dashed lines indicate zero current levels. ***D***, I_{Kr} current densities quantified in control and pentamidine (10μM, 24hr) treated myocytes (n=6-7). * indicates significant difference at $p<0.05$ (Student's t-test).

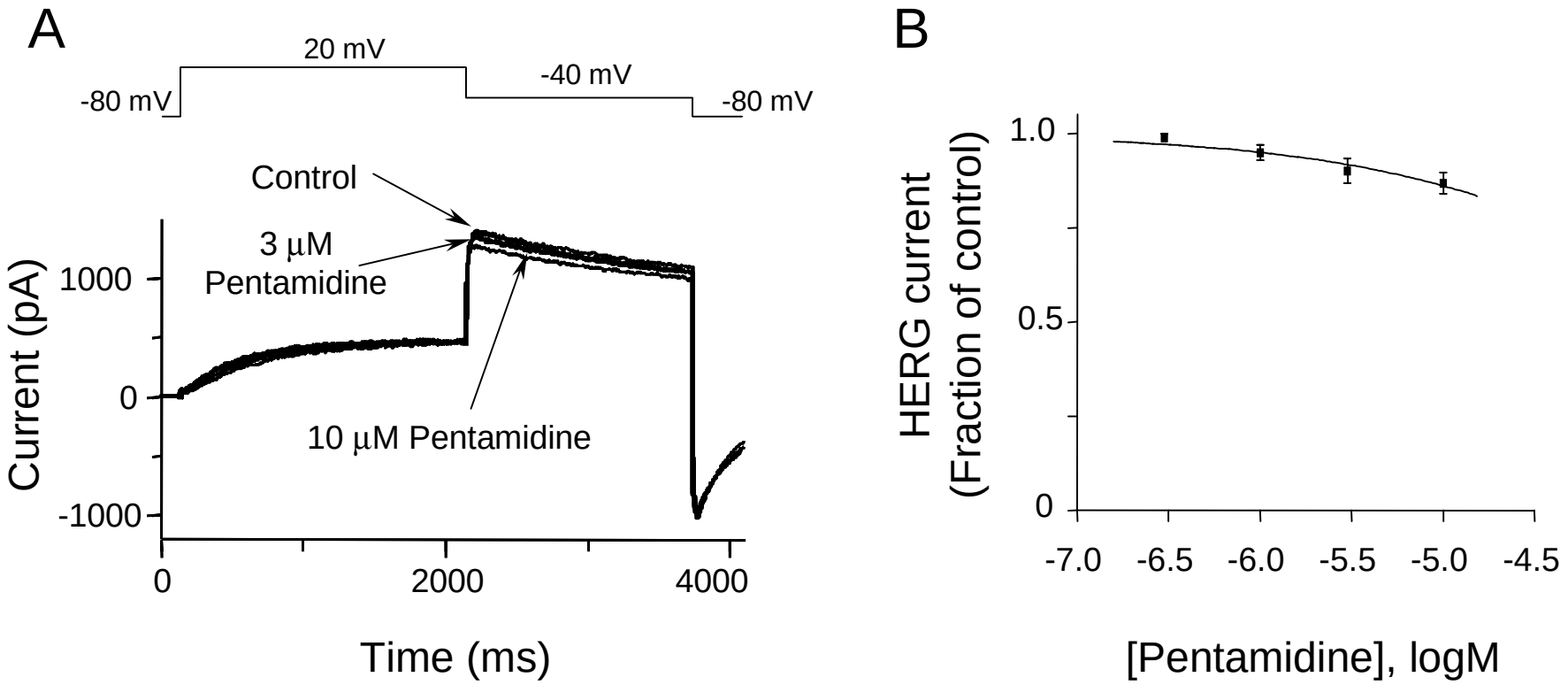

Figure 1

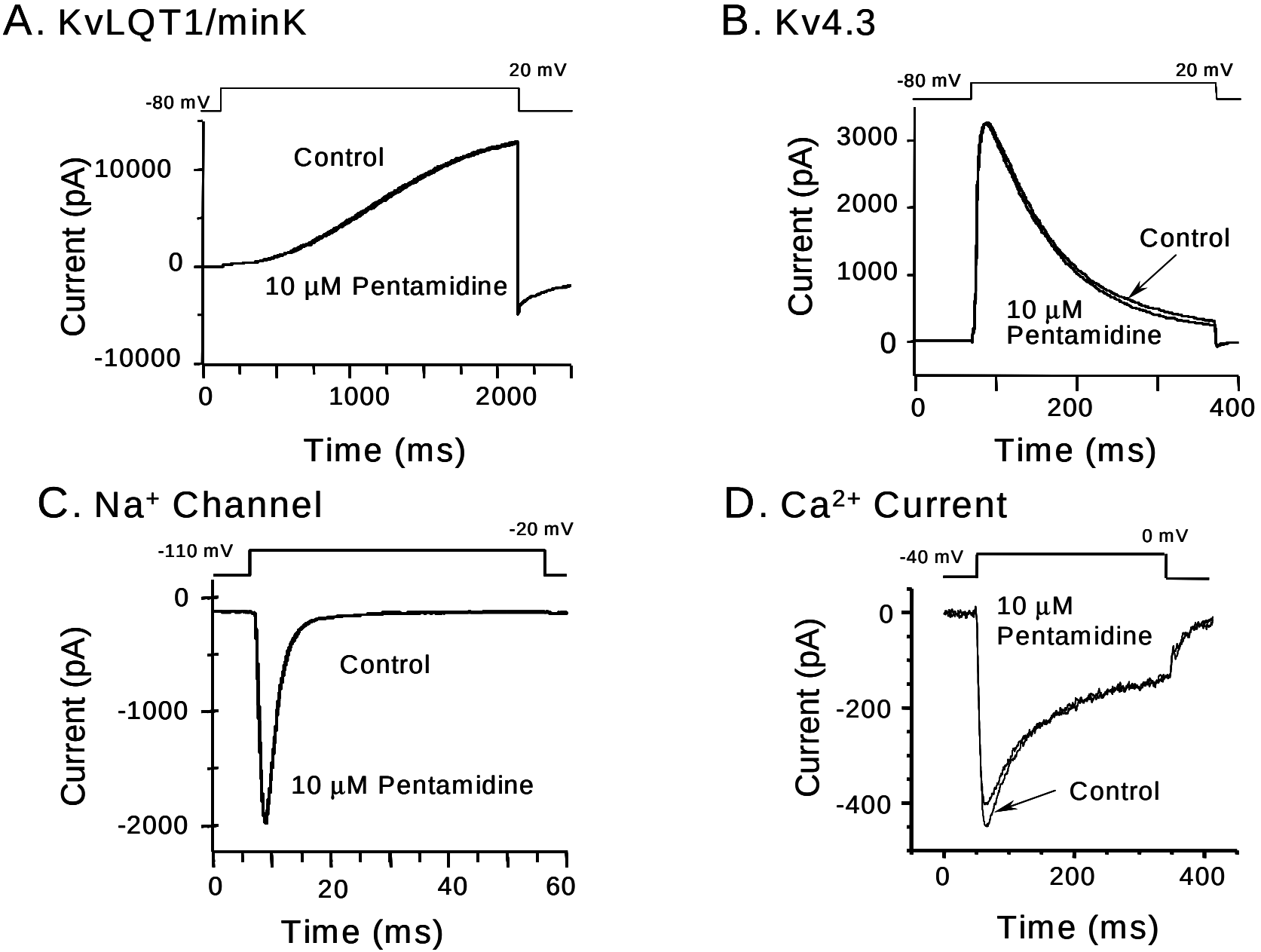

Figure 2

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Figure 3

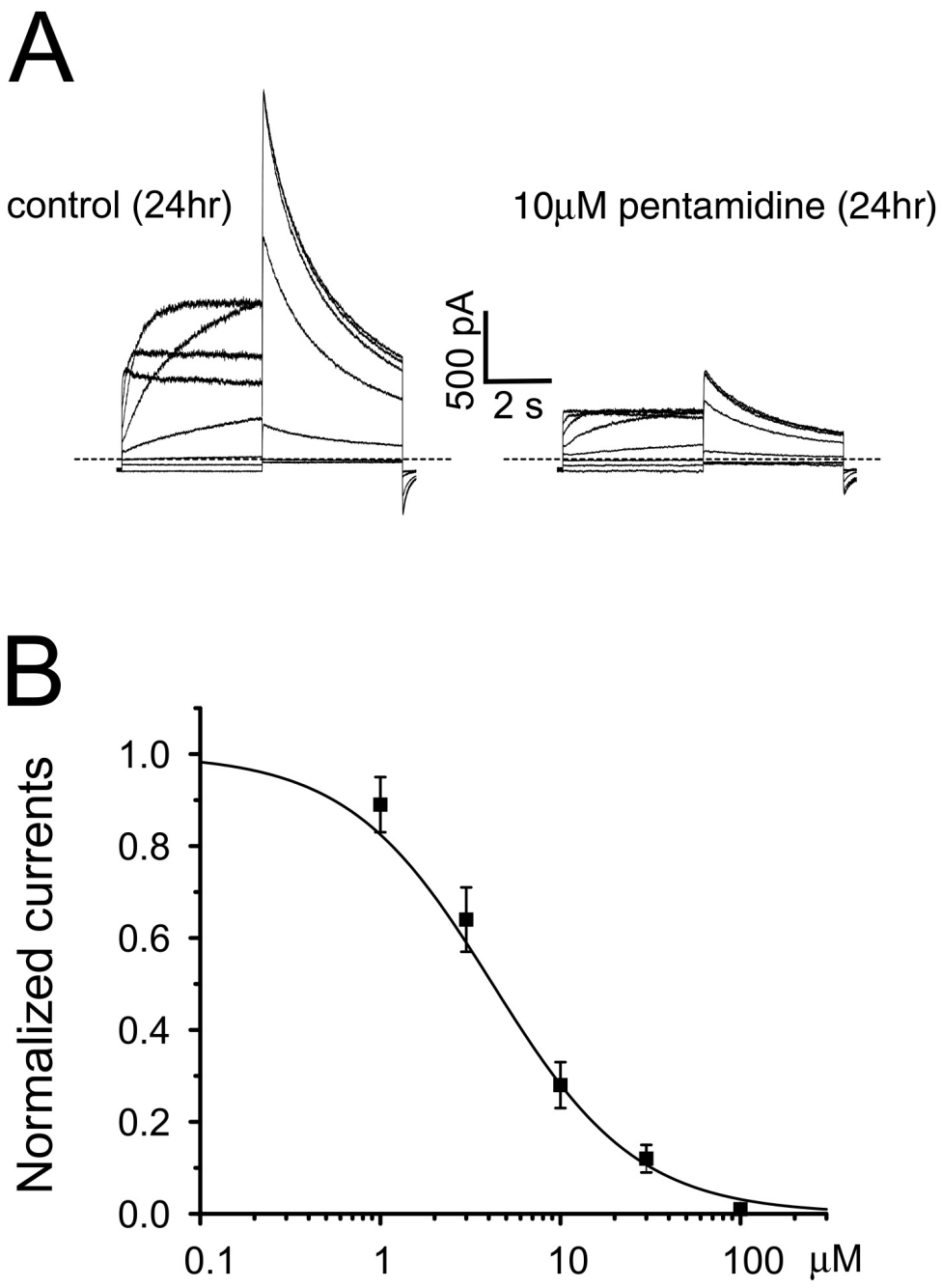

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Figure 4

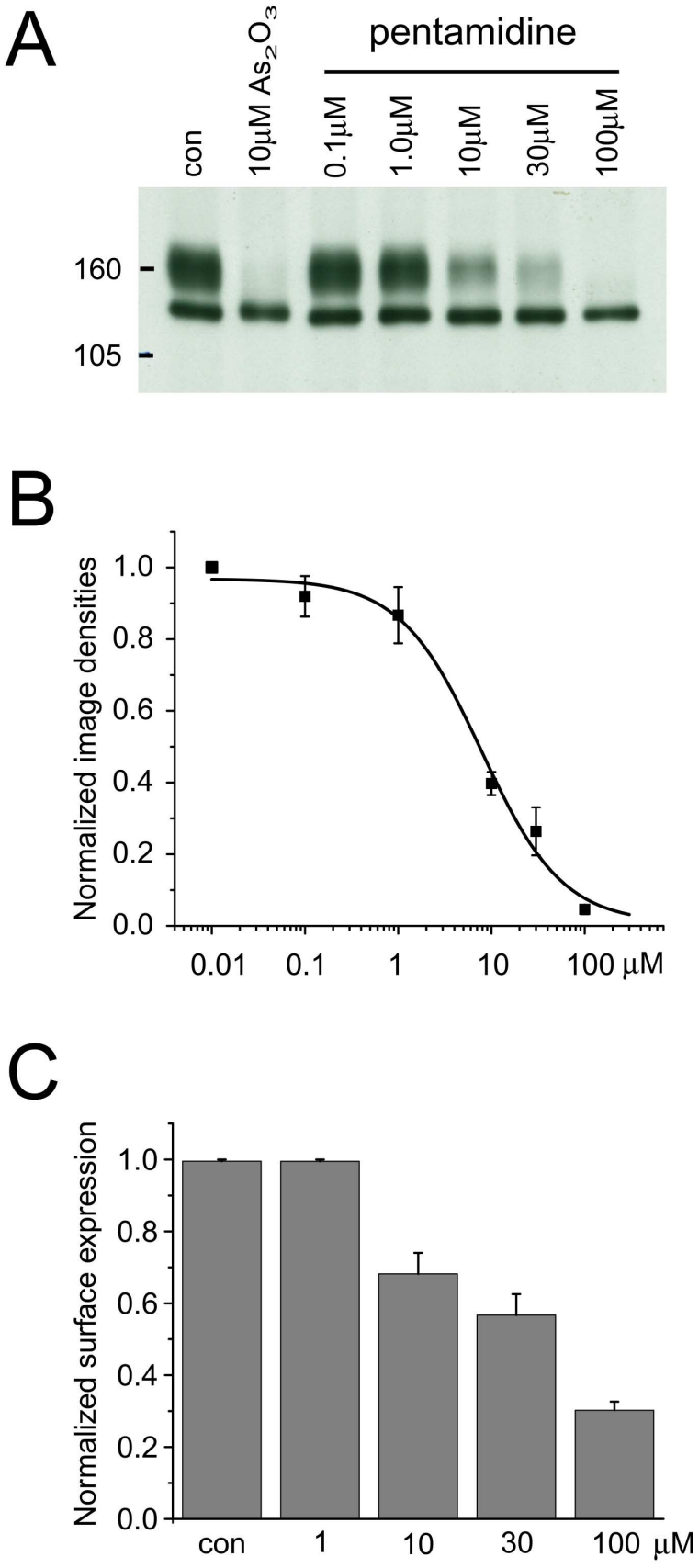

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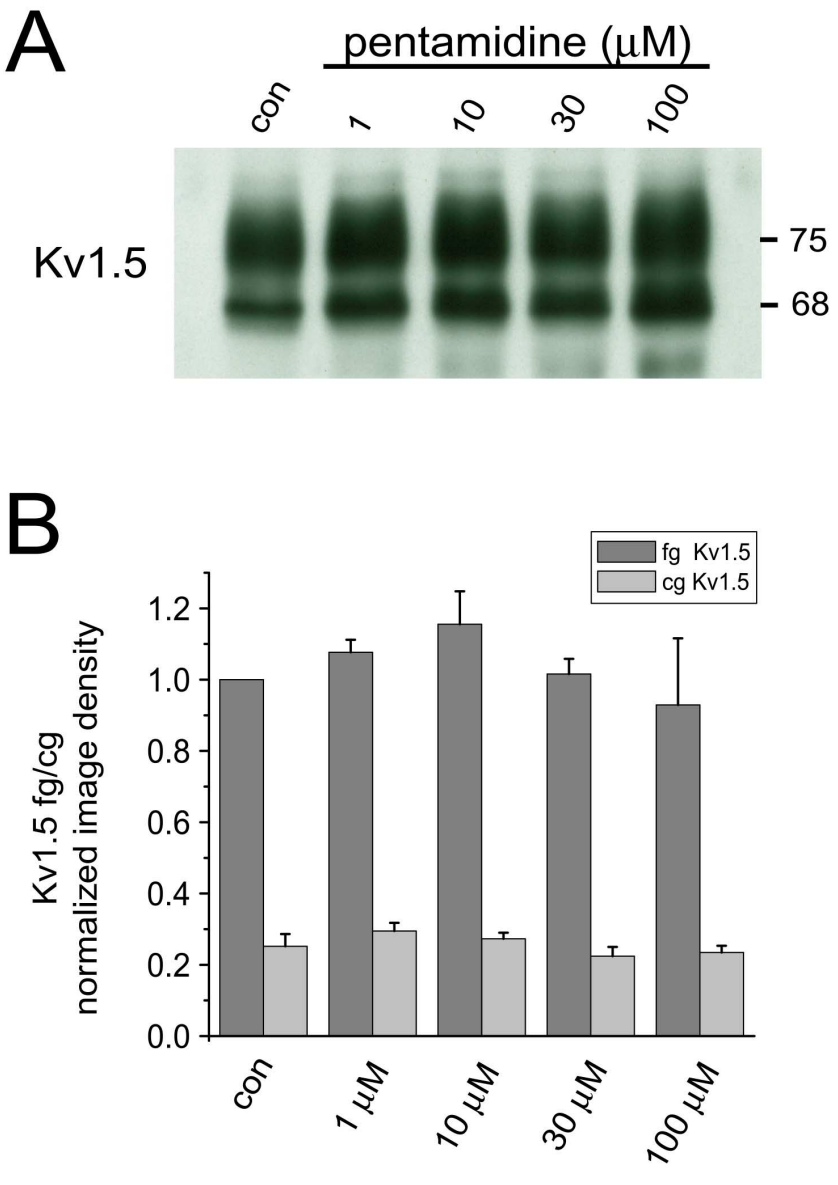
Figure 5



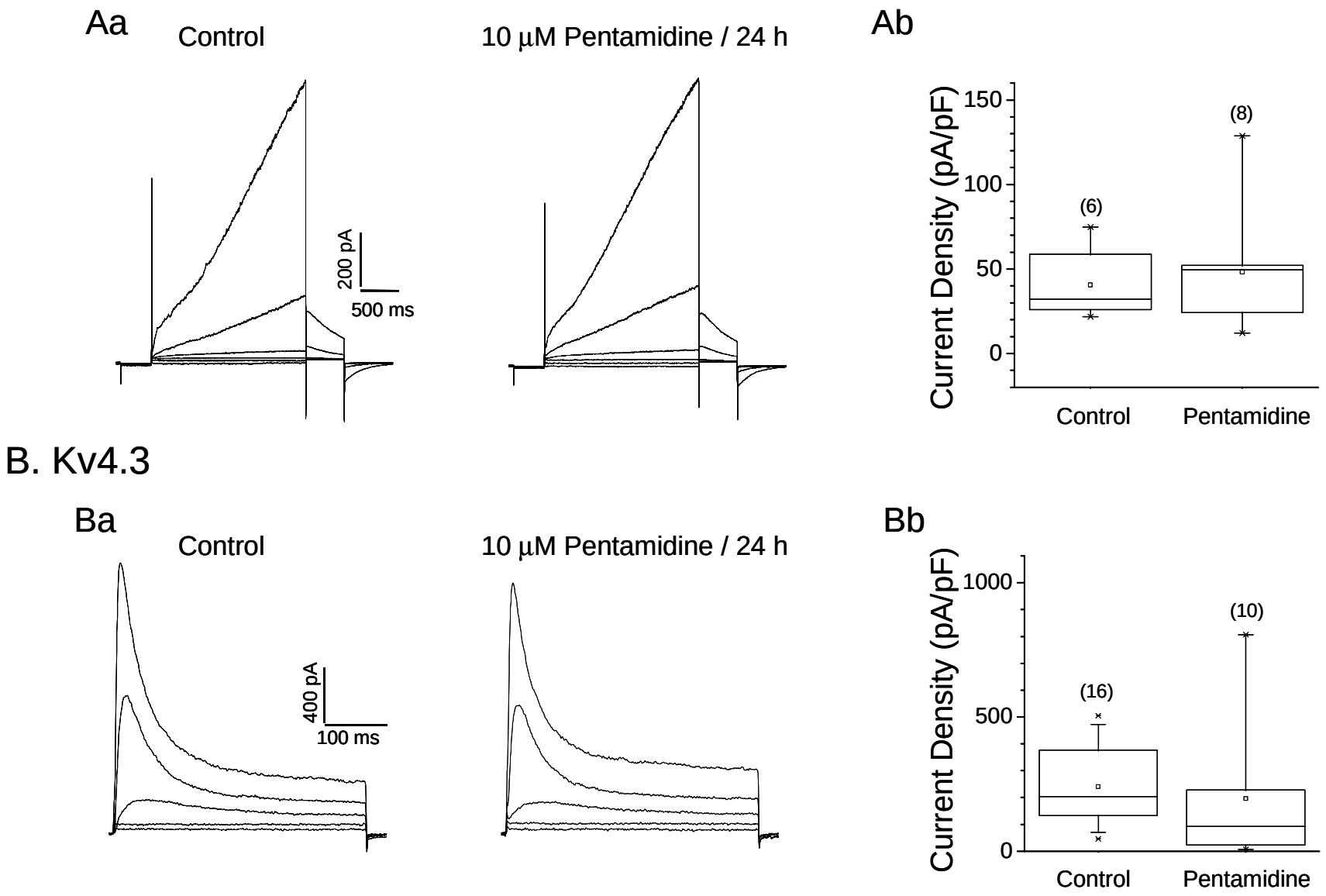


Figure 6

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Figure 7

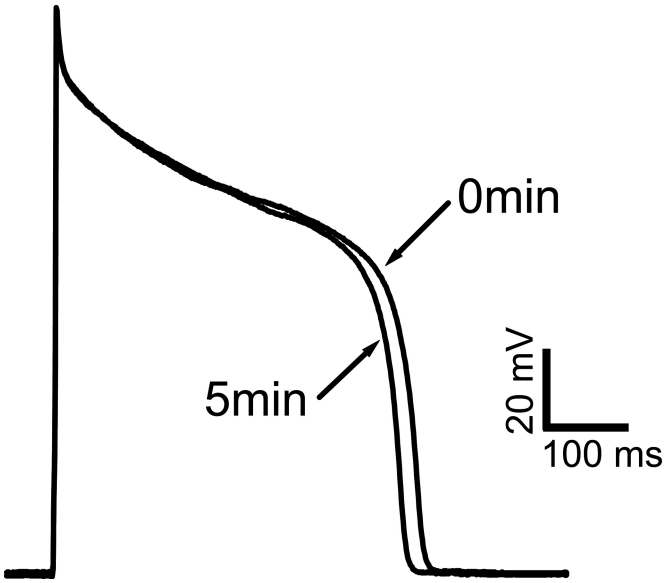

Figure 8